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PRINCIPAL INVESTIGATOR: Richard J. Santen, M.D.

CONTRACTING ORGANIZATION: University of Virginia
                           Charlottesville, VA 22904

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14. ABSTRACT

Deprivation of estrogen, called EndocrineTherapy (ET), is commonly used to treat women with estrogen receptor (ER) positive breast cancer. Resistance to ET occurs in many women after about 18 months of treatment. Upregulation of growth factor pathways mediated by the 21 kD RasGTPase protein may contribute to resistance to ET. A novel Ras antagonist, farnesylthiosalicylate (FTS) causes Ras downreguation with concomitant abrogation of growth factor pathways. We tested the ability of FTS, which was complexed to a cyclodextrin moiety for solubility, to reduce the growth of ER positive breast cancer cells that were resistant to ET. FTS prevented growth of ER positive cells by increasing apoptosis and reducing proliferation. Surprisingly the FTS enhanced apoptosis in breast cancer cells was synergistic with the effects of estradiol. Accompanying loss of cell growth was a significant reduction in the response to estrogen. Of major interest was the unexpected observation that FTS blocked the signaling molecule, mTOR via a novel mechanism, the dissociation of RAPTOR from mTOR. In vivo studies have demonstrated a reduction in tumor size with FTS at doses that caused no demonstrable toxicity. We suggest that FTS should enter preclinical trials against ER positive breast cancer.

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Introduction:

Deprivation of estrogen, called Endocrine Therapy (ET), is commonly used to treat women with estrogen receptor (ER) positive breast cancer. Resistance to ET occurs in a many women after about 18 months treatment. Upregulation of growth factor pathways mediated by the 21 kDa Ras GTPase protein may contribute to resistance to ET. A novel Ras antagonist, farnesylthiosalicylate (FTS), causes Ras downregulation with concomittant abrogation of growth factor pathways. We tested the ability of FTS, which was complexed to a cyclodextrin moiety for solubility, to reduce the growth of ER positive breast cancer cells that were resistant to ET.   FTS prevented growth of ER positive breast cancer cells by increasing apoptosis and reducing proliferation. Accompanying loss of cell growth was a significant reduction in the response to estrogen. The loss of estrogen response may have been due to an observed loss of ER protein in response FTS treatment.   FTS might be causing reduced cell growth in part by increasing turnover of the ER in ER positive breast cancer cells. FTS was additive with Doxorubicin in vitro. The in vivo studies demonstrate that FTS blocks the incorporation of BrdU into DNA in tamoxifen resistant cells.

Body of Report:

Methods

Reagents: FTS and cyclodextrin (CD) were generously donated by Thyreos Corporation, New Jersey. FTS-CD complex was prepared according to instructions from Thyreos; CD alone and PBS buffer controls were prepared exactly the same way. ICI 182,780 was generously donated by Astra-Zeneca, United Kingdom. E2 was obtained from Steraloids (Newport, Rhode Island). Cell Death Detection ELISA and Cell Proliferation ELISA were from Roche. Neutral Red was from Aldrich. IMEM was from Biosource and Fetal Bovine Serum (FBS) from Gibco. DextranT70 was from Pharmacia and Charcoal (NoritA) from Sigma.

Cell Culture: To remove E2 and related metabolites from serum, FBS was treated with Dextran and charcoal. Dextran Charcoal Coated stripped serum (DCC) was prepared. Inactivated FBS (500 ml) was added to 5 g of washed charcoal and 167 mg of DextranT70. The mixture was stirred at 4C overnight and the Dextran/charcoal pelleted out. This was repeated with fresh Dextran/charcoal two more times. In the final step, the FBS was spun twice at 33,000 rpm in an ultracentrifuge and then filtered through a 0.1 µm filter. The resulting Dextran/charcoal coated stripped serum is referred to as DCC.

MCF-7 cells were cultured in IMEM, glutamine and 5% FBS, LTED cells were cultured in phenol red free IMEM, glutamine and 5% DCC. Cell number was assayed by a modified neutral red method. To assay E2 dependent growth, MCF-7 cells were seeded into 96 well plates; the next day the medium was changed to IMEM DCC and 5 days later E2 was added in fresh IMEM/DCC. Three days later fresh E2 in IMEM/DCC was

added and cell number measured 2 days later. To measure response of LTED cells to E2, LTED cells were seeded into 96 well plates; the next day, media was changed to IMEM with just glutamine added. Seven days later, E2 plus 10^{-9} M ICI182,780 in fresh IMEM was added; three days after that fresh E2 was added in IMEM/ICI and 2 days later cell number was measured. To assay the effects of FTS-CD on cell number, proliferation and apoptosis, cells were seeded into 96 well plates and the next day drug was added in fresh medium. Three days later, fresh drug in medium was added and cell number, apoptosis and proliferation measured 2 days later.

Cell Extract Preparation and Western Blotting: Cells were seeded at 2 million cells per 10 cm diameter dish. The next day CD or FTS-CD was added and three days later the cells were harvested into RIPA buffer. Cell extracts were normalized and loaded onto 10% SDS-PAGE gels, transferred to PVDF membrane and probed with the indicated antibodies. The antibodies used were: 62A3 reactive against residues around serine 118 of the ER from Cell Signaling, Ab17 against the N-terminus of the ER, Ab20 against whole ER (both from Neomarkers), phosphoRb against phosphorylated residues 807 and 811 of Rb from Cell Signaling, C-15 against the C-terminus of Rb from Santa Cruz and Actin antibody from Sigma.

Results: (Task I) Breast cancers that are susceptible to Endocrine Therapy are usually ER positive and wild type p53. MCF-7 cells are a wild type p53, ER positive, E2 dependent cell line and are a generally accepted model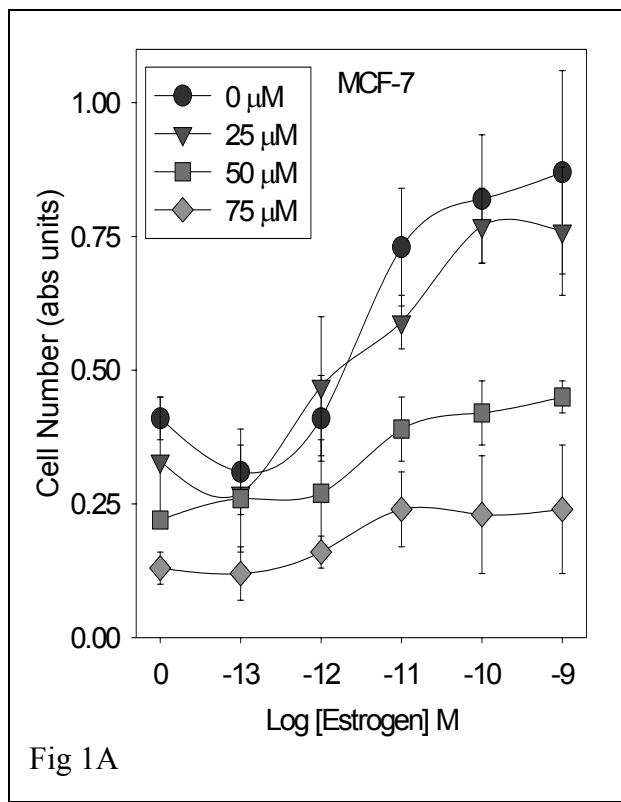 for early stage ER positive breast cancer that is treated by Endocrine Therapy. LTED cells were generated from MCF-7 cells by depriving them of E2 over several months. LTED cells have many of the characteristics of breast cancers that regrow in the low E2 serum levels after Endocrine Therapy. Upregulation of growth factor and ER pathways occurs during regrowth after Endocrine Therapy. Therefore we tested whether a growth factor pathway inhibitor suppressed the growth of breast cancer cells in vitro. We assayed the effect of FTS, a Ras inhibitor, as the 21 kDa Ras protein is at the nexus of several important growth factor pathways, as well as being implicated in plasma membrane based ER signaling FTS suppressed the E2 dependent growth of MCF-7 cells (Figure 1A) with the greatest effects at 25 and 75 μM.

Fig 1A

Figure 1: FTS suppresses E2 dependent breast cancer cell growth. MCF-7 (**A**) and LTED

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(**B**) cells were grown under conditions where they demonstrate an E2 response as described in the methods. Increasing concentrations of FTS suppressed this E2 dependence. Representative of 2 experiments, mean and standard deviation of 4 samples are shown.

LTED cells display the characteristic maximal <u>hypersensitive</u> growth at two log lower E2 concentration than MCF-7 cells (Figure 1B). FTS started suppressing LTED growth at 25 μM and there was a paradoxical slight stimulation of growth at 75 μM FTS. There was little growth of either cell line in the absence of E2 under these conditions. These data support previous observations that E2 dependent ER signaling can pass through Ras *

We next compared free FTS to FTS complexed with CD. Complexing FTS with CD is required to solubilize the hydrophobic FTS molecule and would be essential for any possible future clinical use of FTS. FTS and FTS-CD were very similar in inhibiting cell growth of MCF-7 cells (Figure 2A). CD alone or Buffer vehicle had little effect on cell growth of MCF-7 and LTED cells (Figures 2B and 2C) under conditions where FTS-CD significantly abrogated cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells.
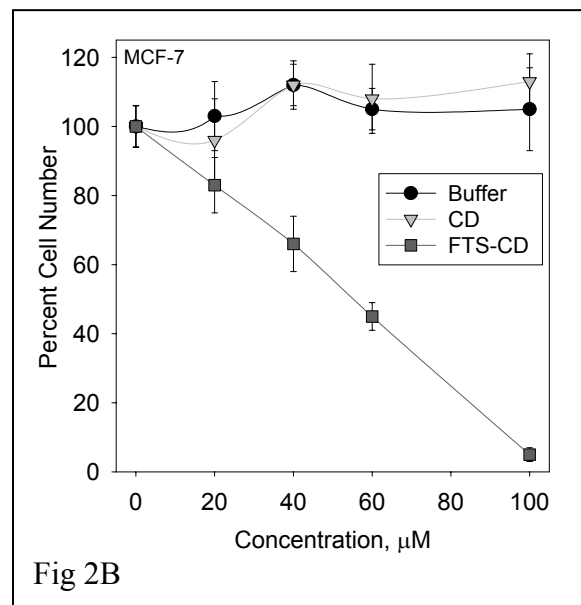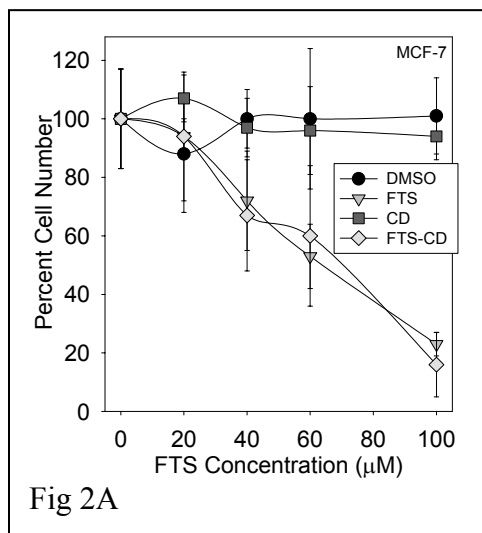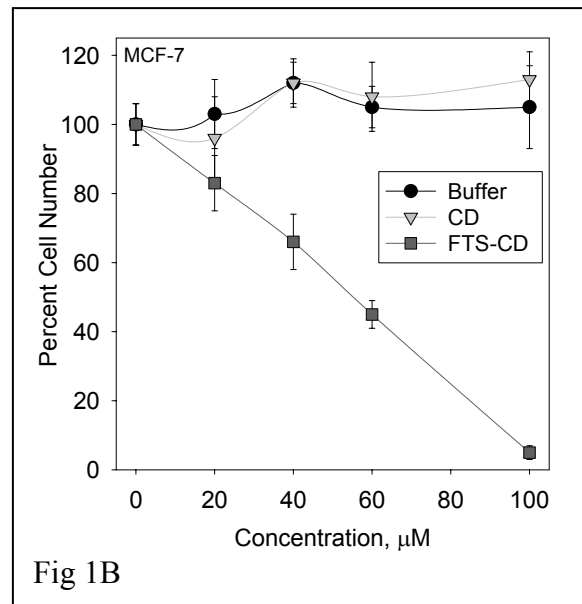
Fig 1B

Fig 2A

Fig 2B

Figure 2: FTS and FTS-CD have similar growth inhibition profiles. **A**. MCF-7 cells were grown in the presence of FTS dissolved in DMSO, the equivalent volume of DMSO, FTS-CD dissolved in PBS or the equivalent amount of CD in PBS. Cells were treated for 5 days according to the methods. Representative of 2 experiments, mean and standard deviation of 4 samples are shown. **B**. and **C**. FTS-CD inhibits growth of both MCF-7 (**B**) and LTED (**C**) cells. Cells were

assayed according to the methods with either PBS, CD in PBS or FTS-CD in PBS added. Representative of 6 experiments, mean and standard deviation of 4 samples are shown.

Ras activity is required for cellular proliferation. Either underexpression or overexpression of Ras is capable of inducing an apoptotic response Apoptosis was induced by between several hundred (not shown) to several thousand fold (Figures 3A and 3B) by 100 μM FTS-CD, which is known to decrease the amount of cellular Ras. Proliferation was also reduced to very low amounts in both cell lines by FTS-CD, but not by CD or buffer alone (Figures 4A and 4B), affirming a reduction in an essential component of proliferative pathways.
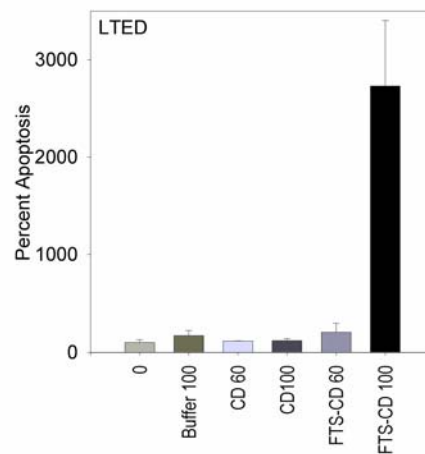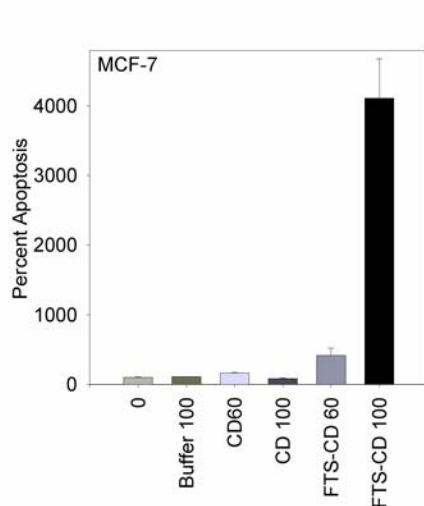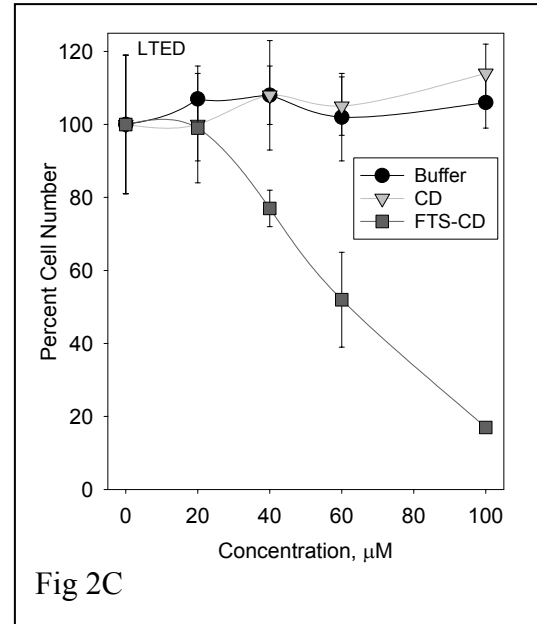
Fig 2C

Fig 3A

Fig 3B

Figure 3: FTS-CD increases apoptosis. MCF-7 (**A**) and LTED (**B**) cells were incubated with buffer (equivalent to 100 μM FTS-CD), CD (equivalent to 60 or 100 μM FTS-CD) or 60 or 100 μM FTS-CD for five days as described in Methods. Apoptosis was measured by DNA nick site quantitative by ELISA. Representative of two experiments, mean and standard deviation of two samples are shown.
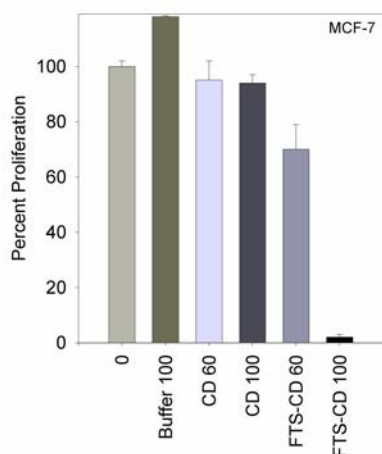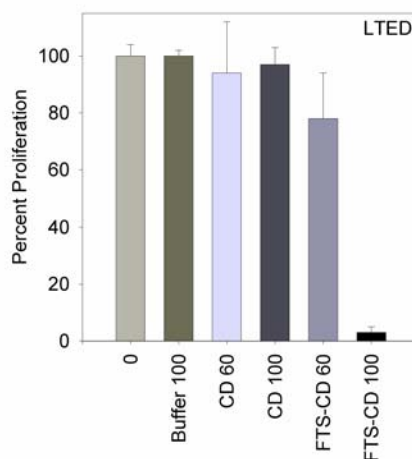
Fig 4A   Fig 4B

Figure 4: FTS-CD reduces proliferation. MCF-7 (**A**) and LTED (**B**) cells were incubated with buffer, CD or FTS-CD for five days as described in Figure 3 legend. Proliferation was measured by BrDU incorporation. Representative of two experiments,   mean and standard deviation of two samples are shown.

       Downregulation of the ER is known to block E2 responsiveness, induce apoptosis and decrease proliferation of breast cancer cells. ER protein levels were downregulated after one to three days of FTS-CD treatment (Figure 5). This was confirmed with three different antibodies against different regions of the ER.
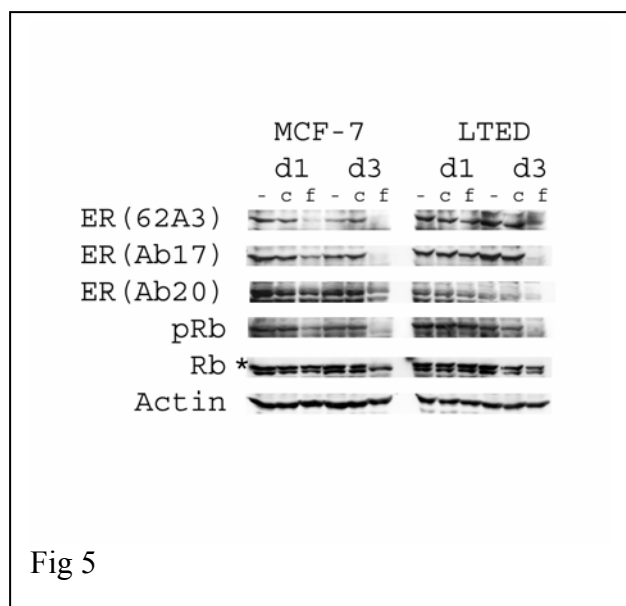

Fig 5

Figure 5: FTS-CD causes loss of the ER. MCF-7 (left six lanes) and LTED (right six lanes) cells were incubated with 100 μM FTS-CD (f) or CD equivalent (c) or vehicle (-) for one (d1) or three (d3) days. The cells were harvested and proteins separated by SDS-PAGE, transferred to PVDF membrane and probed with indicated antibodies. Note that the Rb specific band is the upper of the two bands shown, asterixed. The lower Rb band appears to be non specific and does not align with the phosphoRb bands. Representative of two experiments.

       Our data support a hypothesis that a major mechansism of FTS-CD on breast cancer cells is abrogation of E2 signaling, possibly by down-regulation of the ER protein.   The tumor suppressor Rb is phosphorylated in cells

stimulated by E2 . Phosphorylation of Rb allows activation of E2F1 and progression of the cell cycle. Rb phosphorylation was reduced by FTS-CD after three days (Figure 5). We also noted a decrease in total Rb in MCF-7 cells after 3 days of FTS-CD treatment but no decrease was observed in LTED cells.

The ability of FTS-CD to synergize with established anti-cancer drugs was tested. Notably Doxorubicin was additive with FTS-CD (Figure 6). Additivity occurred after 6 days growth at relatively low cell concentrations of 500 cells seeded per 96 plate well, but was not as marked at higher seeding densities of 750 and 1000 cells per well (not shown). We also tested additivity of FTS-CD with Tamoxifen, Paclitaxel and Cyclophosphamide prodrug but did not observe any reproducible additivity under the conditions used (not shown).
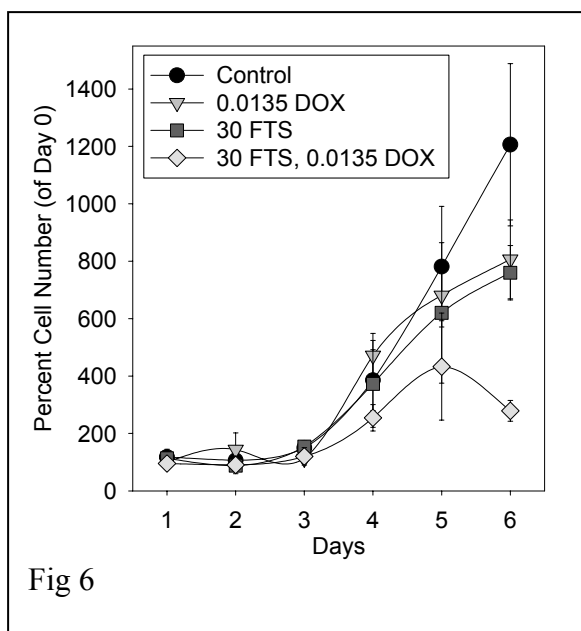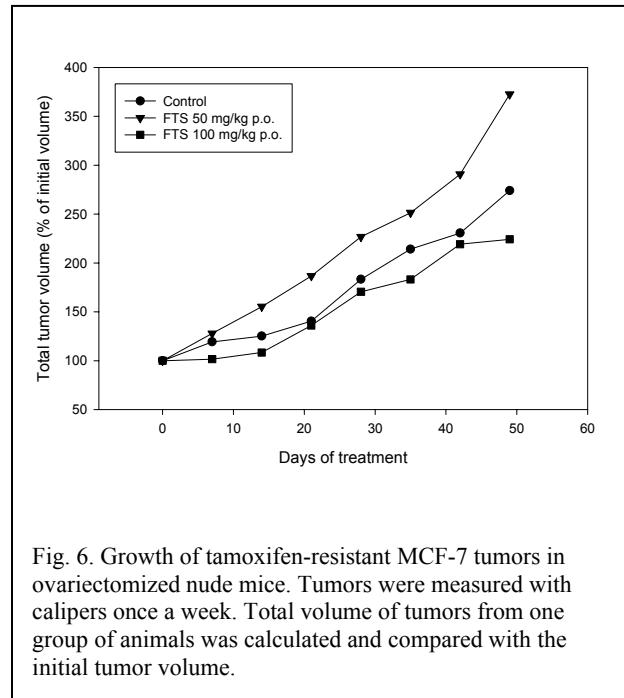

Fig 6

Figure 6: FTS-CD is additive with Doxorubicin. MCF-7 cells were seeded at 500 cells per well of a 96 well plate. The next day (day 0) cell number was measured as in methods and FTS-CD (30 mM) and/or Doxorubicin (0.0135 μM) were added to the remaining wells as in the legend. Cell number was measured every day and fresh drug and media were added at Day 3. Shown are mean and standard deviation of four wells, expressed as a percentage of the number of cells measured at day 0. Representative of three experiments.
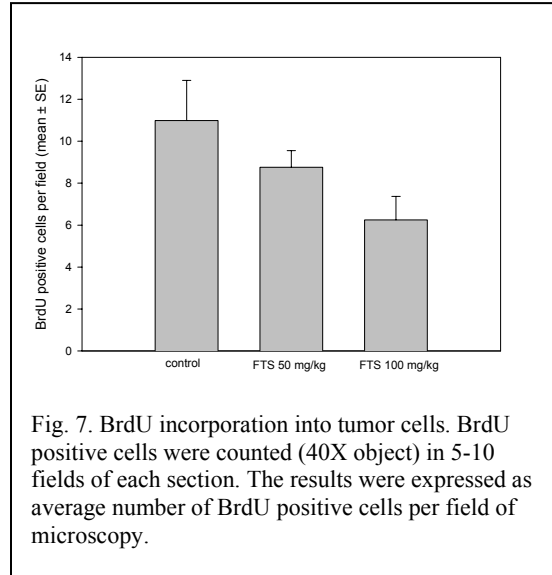
Results Task II

Tumors originally developed from MCF-7 breast cancer cells in nude mice were exposed to tamoxifen and developed resistance after serial transplantations in animals for up to five years. These tumors can grow in ovariectomized mice with or without tamoxifen supplement. This model is considered a model of acquired resistance to endocrine therapy. We transplanted a piece of 1 mm^3 tumor tissue under the skin of ovariectomized nude mice and allowed the tumor to establish without any supplement. The animals were randomly divided into three groups that were assigned to receive vehicle (corn oil, 2.5 ml/kg) or FTS at two doses (50 mg/kg and 100 mg/kg, po). Seven weeks later, animals were injected with BrdU IP and sacrificed 2 hours after injection. Tumor tissues were fixed in 10% formalin in PBS or snap frozen in liquid nitrogen. Formalin-fixed tissues were sectioned and BrdU incorporation was determined by immunostaining with specific anti-BrdU antibody followed by AP-conjugated secondary antibody using a Cell

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Proliferation Kit from the Roche. Frozen tissues were homogenized in lyses buffer for western analysis.

FTS administered orally at 50 mg/kg daily slightly increased tumor growth rate. There was no significant affect on tumor growth with higher dose of FTS (Fig. 6).

Fig. 6. Growth of tamoxifen-resistant MCF-7 tumors in ovariectomized nude mice. Tumors were measured with calipers once a week. Total volume of tumors from one group of animals was calculated and compared with the initial tumor volume.

Although there was no significant reduction in tumor growth rate, tumors from FTS treated mice displayed lower proliferation rate measured by BrdU incorporation (Fig. 7).

Fig. 7. BrdU incorporation into tumor cells. BrdU positive cells were counted (40X object) in 5-10 fields of each section. The results were expressed as average number of BrdU positive cells per field of microscopy.

Western analysis of tumor lysates was also carried out. No inhibition but stimulation of MAPK phosphorylation was detected in tumors from FTS treated animals. While FTS seemed not to increase PARP cleavage, a marker of apoptosis, Bcl2 levels were reduced in the tumors from animals receiving FTS. These results suggest FTS might affect cell survival in vivo.
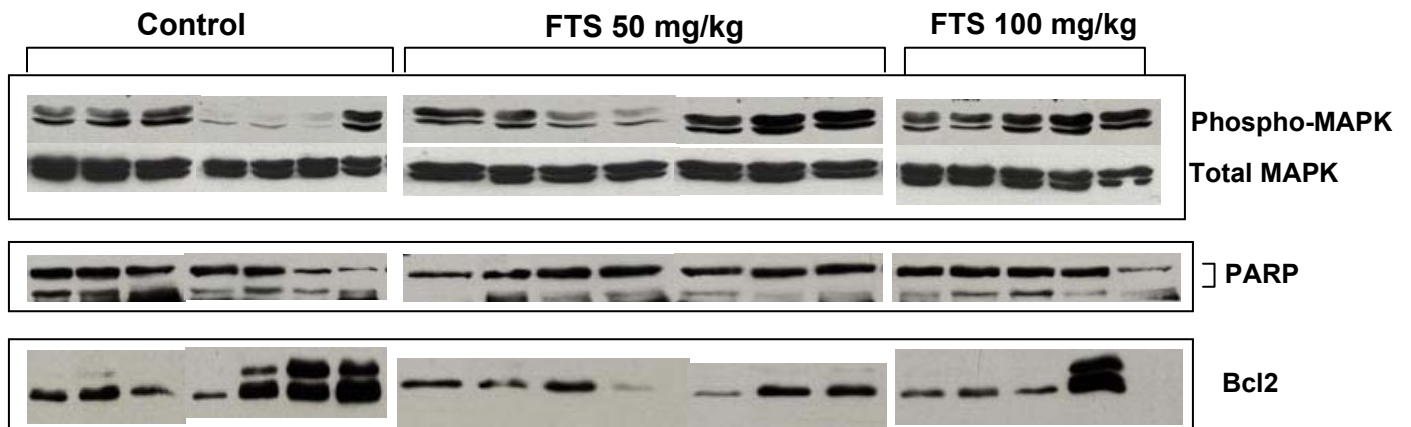

Fig. 8. Western analysis of MAPK, PARP, and Bcl2.

Key Research Accomplishments

The soluble FTS-CD complex clearly prevents growth of cellular models of E2 dependent (MCF-7) and E2 hypersensitive (LTED) breast cancers. The compound induces apoptosis up to several thousand fold and reduces proliferation to very low levels. The LTED cells have upregulated MAPK activity compared to parental MCF-7 cells addition of a specific MAPK inhibitor to LTED cells changes the E2 concentration that stimulates maximal cell growth. That is, they become less sensitive to E2 but growth is not completely suppressed. The FTS-CD suppresses growth but does not markedly shift E2 sensitivity. If the main effect of FTS-CD were solely mediated by MAPK, then the LTED cells with higher MAPK activity should have altered sensitivity to FTS-CD. The LTED and MCF-7 cells have the same sensitivity to FTS-CD so we conclude that a major cellular effect of FTS-CD is not through MAPK, but is on other pathways even though FTS-CD does change MAPK activity substantially in these cells. An exception to our hypothesis is the apparent mild shift in hypersensitivity of LTED cells at 75 μM FTS-CD. This observation implies that maximal inhibition of Ras activity in LTED cells, which have hyperactivated MAPK, might partially restore E2 sensitivity. Because we do not know why MAPK activity is upregulated in LTED cells, this anomolous observation is difficult to explain. However LTED cells do have more ER than parental MCF-7 cells, and it is possible that there is simply a different response because of this.

The observation that FTS-CD suppresses E2 dependent growth but does not shift hypersensitivity may be significant. Suppression of ER mediated growth suggested that ER signaling was being suppressed, rather than modified. Modification of ER responsiveness has been observed with the specific MAPK inhibitor, U0126, which changes the maximal E2 response of ER Decreased levels of ER protein suggest the suppression of the E2 response might be though increased ER turnover. ER protein is normally degraded by proteasomal mechanisms and proteasomal inhibitors are very effective inhibitors of breast cancer cell growth in vitro (McPherson unpub.). The proteasomal pathway can be activated by oncogenic hyper activated Ras under certain conditions. Under other conditions, hyperactivated Ras can inhibit the proteasomal degradation of myc protein. Our observation that inhibition of Ras activates degradation pathways is consistent with a role for Ras as an inhibitor of proteasomal pathways in ER positive breast cancer cells. The alternative explanation for reduced ER levels on FTS-CD treatment is a reduction in transcription/translation of the ER. This hypothesis is also attractive because the ER has a half life of only a few hours Our current data do not allow us to discriminate between these two possibilities.

Anthracyclines have a survival benefit of 3% over non-anthracycline chemotherapies of breast cancer at five years. FTS-CD is reproducibly additive with the anthracycline Doxorubicin in vitro. However this additivity was maximal at relatively lower cell densities. At lower cell densities there is less autocrine stimulation of Ras and MAPK, so pathway activity is lower than at higher cell densities. In cancer cells a

relatively high density might occur as cells aggregate inappropriately. Whether this combination of drugs will be effective against breast cancer in vivo remains to be tested. FTS has not had significant toxicity in numerous preclinical trials. FTS is effective against breast cancer cells in vitro and is additive with an established anti-cancer drug. Therefore we suggest FTS-CD should undergo preclinical trials against breast cancer.

The in vivo studies demonstrated that FTS blocked the incorporation of BrdU into DNA but did not result in a decrease in tumor growth. These studies demonstrated a positive effect but the lack of tumor growth inhibition is puzzling. It is possible that apoptosis may be effected differentially from proliferation in the in vivo model but the data available at present does not allow us to make this conclusion.

Reportable Outcomes: The data on in vitro inhibition of breast cancer cell growth. This work has now been published. (Santen RJ, Lynch AR, Neal LR, McPherson RA, Yue W. **Farnesylthiosalicylic acid: inhibition of proliferation and enhancement of apoptosis of hormone-dependent breast cancer cells Anticancer Drugs 17:33-40, 2006**

Conclusions: These data provide compelling evidence that FTS causes a blockade of cell proliferation and an increase in apoptosis in breast cancer cells. These data show a high degree of promise that this agent will be effective in patients with breast cancer.

Bibliography:

1. Leonessa F, Boulay V, Wright A, Thompson EW, Brunner N, Clarke R :**The biology of breast tumor progression. Acquisition of hormone independence and resistance to cytotoxic drugs**. *Acta Oncol* 1992, **31**:115-123.
2. Santen RJ and Harvey HA :**Use of aromatase inhibitors in breast carcinoma**. *Endocr Relat Cancer* 1999, **6**:75-92.
3. Santen RJ and Harvey HA :**Use of aromatase inhibitors in breast carcinoma**. *Endocr Relat Cancer* 1999, **6**:75-92.
4. Santen RJ, Manni A, Harvey H, Redmond C :**Endocrine treatment of breast cancer in women**. *Endocr Rev* 1990, **11**:221-265.
5. Osborne CK :**Aromatase inhibitors in relation to other forms of endocrine therapy for breast cancer**. *Endocr Relat Cancer* 1999, **6**:271-276.
6. Gradishar WJ and Jordan VC :**Hormonal therapy for breast cancer. An update**. *Hematol Oncol Clin North Am* 1999, **13**:435-55, vii.
7. Hortobagyi GN :**Treatment of breast cancer [see comments]**. *N Engl J Med* 1998, **339**:974-984.
8. EarlyBreastCancerTrialists' :**Ovarian ablation for early breast cancer. Early Breast Cancer Trialists' Collaborative Group**. *Cochrane Database Syst Rev* 2000, **2**:.
9. Nicholson RI and Gee JM :**Oestrogen and growth factor cross-talk and endocrine insensitivity and acquired resistance in breast cancer**. *Br J Cancer* 2000, **82**:501-513.
10. Gottardis MM, Wagner RJ, Borden EC, Jordan VC :**Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro**. *Cancer Res* 1989, **49**:4765-9..
11. de Cupis A and Favoni RE :**Oestrogen/growth factor cross-talk in breast carcinoma: a specific target for novel antioestrogens [published erratum appears in Trends Pharmacol Sci 1997 Sep;18(9):345-6]**. *Trends Pharmacol Sci* 1997, **18**:245-251.
12. Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, Yue W :**The role of mitogen-activated protein (MAP) kinase in breast cancer**. *J Steroid Biochem Mol Biol* 2002, **80**:239-56..
13. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F :**Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation**. *Embo J* 2000, **19**:5406-17..
14. Rosner W, Hryb DJ, Khan MS, Nakhla AM, Romas NA :**Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane**. *J Steroid Biochem Mol Biol* 1999, **69**:481-5..
15. McCormick F :**Signalling networks that cause cancer**. *Trends Cell Biol* 1999, **9**:M53-6.
16. Gibbs JB, Graham SL, Hartman GD, Koblan KS, Kohl NE, Omer CA, Oliff A :**Farnesyltransferase inhibitors versus Ras inhibitors**. *Curr Opin Chem Biol* 1997, **1**:197-203.
17. Conti CJ :**Mutations of genes of the ras family in human and experimental tumors**. *Prog Clin Biol Res* 1992, **376**:357-378.

18. Casey PJ :**Protein lipidation in cell signaling**. *Science* 1995, **268**:221-225.
19. Cox AD and Der CJ :**Farnesyltransferase inhibitors: promises and realities**. *Curr Opin Pharmacol* 2002, **2**:388-93..
20. Sebti S and Hamilton AD :**Inhibitors of prenyl transferases**. *Curr Opin Oncol* 1997, **9**:557-561.
21. Kloog Y, Cox AD, Sinensky M :**Concepts in Ras-directed therapy**. *Exp. Opin. Invest. drugs* 1999, **8**:212-2140.
22. Niv H, Gutman O, Henis YI, Kloog Y :**Membrane interactions of a constitutively active GFP-Ki-Ras 4B and their role in signaling. Evidence from lateral mobility studies**. *J Biol Chem* 1999, **274**:1606-1613.
23. Eskens FA SG, Verweij J. :**Farnesyl transferase inhibitors: current developments and future perspectives**. *Cancer Treat Rev* 2000, **26**:319-332.
24. Smith V, Rowlands MG, Barrie E, Workman P, Kelland LR :**Establishment and characterization of acquired resistance to the farnesyl protein transferase inhibitor R115777 in a human colon cancer cell line**. *Clin Cancer Res* 2002, **8**:2002-2009.
25. Gana-Weisz M, Halaschek-Wiener J, Jansen B, Elad G, Haklai R, Kloog Y :**The Ras inhibitor S-trans,trans-farnesylthiosalicylic acid chemosensitizes human tumor cells without causing resistance**. *Clin Cancer Res* 2002, **8**:555-65..
26. Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Kmar R, Santen RJ :**Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo**. *Endocrinology* 2000, **141**:396-405.
27. Masamura S, Santner SJ, Heitjan DF, Santen RJ :**Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells**. *J Clin Endocrinol Metab* 1995, **80**:2918-2925.
28. Jeng MH, Yue W, Eischeid A, Wang JP, Santen RJ :**Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells**. *Breast Cancer Res Treat* 2000, **62**:167-75..
29. Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Kmar R, Santen RJ :**Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo**. *Endocrinology* 2000, **141**:396-405.
30. Horwitz KB and McGuire WL :**Nuclear mechanisms of estrogen action. Effects of estradiol and anti-estrogens on estrogen receptors and nuclear receptor processing**. *J Biol Chem* 1978, **253**:8185-8191.
31. Fiennes AG, Walton J, Winterbourne D, McGlashan D, Hermon-Taylor J :**Quantitative correlation of neutral red dye uptake with cell number in human cancer cell cultures**. *Cell Biol Int Rep* 1987, **11**:373-8..
32. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Bottero D, Varricchio L, Nanayakkara M, Rotondi A, Auricchio F :**Sex steroid hormones act as growth factors**. *J Steroid Biochem Mol Biol* 2002, **83**:31-35.
33. Duan R, Xie W, Li X, McDougal A, Safe S :**Estrogen regulation of c-fos gene expression through phosphatidylinositol-3-kinase-dependent activation of serum response factor in MCF-7 breast cancer cells**. *Biochem Biophys Res Commun* 2002, **294**:384-394.

34. Cox AD and Der CJ :**The dark side of Ras: regulation of apoptosis**. *Oncogene* 2003, **22**:8999-9006.
35. Li C, Chi S, He N, Zhang X, Guicherit O, Wagner R, Tyring S, Xie J :**IFNalpha induces Fas expression and apoptosis in hedgehog pathway activated BCC cells through inhibiting Ras-Erk signaling**. *Oncogene* 2003, .
36. Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, Weisz A :**17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells**. *Oncogene* 1996, **12**:2315-2324.
37. Yue W, Wang JP, Conaway MR, Li Y, Santen RJ :**Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways**. *J Steroid Biochem Mol Biol* 2003, **86**:265-274.
38. Saha D, Datta PK, Beauchamp RD :**Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4**. *J Biol Chem* 2001, **276**:29531-29537.
39. Shao J, Sheng H, DuBois RN, Beauchamp RD :**Oncogenic Ras-mediated cell growth arrest and apoptosis are associated with increased ubiquitin-dependent cyclin D1 degradation**. *J Biol Chem* 2000, **275**:22916-22924.
40. Sears R, Leone G, DeGregori J, Nevins JR :**Ras enhances Myc protein stability**. *Mol Cell* 1999, **3**:169-179.
41. Nardulli AM and Katzenellenbogen BS :**Dynamics of estrogen receptor turnover in uterine cells in vitro and in uteri in vivo**. *Endocrinology* 1986, **119**:2038-2046.
42. Bergh J, Jonsson PE, Glimelius B, Nygren P, CN - SSCoTAiHC :**A systematic overview of chemotherapy effects in breast cancer**. *Acta Oncol* 2001, **40**:253-281.

Farnesylthiosalicylic acid: inhibition of proliferation and enhancement of apoptosis of hormone-dependent breast cancer cells

Richard J Santen[a], Amanda R Lynch[a], Lindsey R Neal[a], Robert A McPherson[a,*] and Wei Yue[a]

Farnesyltransferase inhibitors (FTIs) are being developed to block Ras-mediated actions, but current data suggest that the FTIs act through other non-Ras pathways. A new agent, farnesylthiosalicylic acid (FTS), blocks the binding of Ras to membrane acceptor sites and causes a marked reduction in Ras levels. Accordingly, FTS could be a useful new agent for the treatment of hormone-dependent breast cancer. We examined the dose–response effects of FTS on the growth of MCF-7 breast cancer cells *in vitro* and *in vivo*. Further, we dissected out its specific effects on cell proliferation and apoptosis by measuring BrdU incorporation into DNA and by using an ELISA assay to quantitate the magnitude of apoptosis. FTS and its solubilized conjoiner FTS–cyclodextrin markedly inhibited cell growth in MCF-7 breast cancer cells in culture and in xenografts. This agent exerted dual effects to reduce cell proliferation as assessed by BrdU incorporation and to enhance apoptosis as quantitated by ELISA assay. These data suggest that FTS is a promising agent to be developed for treatment of hormone-dependent breast cancer. *Anti-Cancer Drugs* 17:33–40 © 2006 Lippincott Williams & Wilkins.

[a]Division of Endocrinology, University of Virginia Health Sciences Center, Charlottesville, Virginia, USA.

Correspondence to R.J. Santen, University of Virginia Health Sciences Center, PO Box 801416, Charlottesville, VA 22908, USA.
Tel: +1 434 924 2961; fax: +1 434 924 1284;
e-mail: rjs5y@virginia.edu

Introduction

One-third of human breast cancers depend upon estrogen for growth and regress upon exposure to anti-estrogens or inhibitors of estrogen biosynthesis (e.g. aromatase inhibitors) [1]. In advanced breast cancer, initial responses to the anti-estrogen tamoxifen last for 12–18 months on average, but tumors nearly uniformly begin to regrow later [1,2]. Secondary therapies with aromatase inhibitors often cause additional tumor regressions, but are again followed by relapse [1]. Extensive recent work has focused upon the mechanisms underlying relapse during tamoxifen or aromatase inhibitor therapy [3–5]. These studies demonstrate upregulation of growth factor pathways involving the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling cascades [5–9]. Based upon this concept, a number of investigative groups have suggested that growth factor inhibitors might serve as ideal agents to prolong responses to hormonal therapy in breast cancer or to control progression into an estrogen-independent state [5,9–12].

Binding of a number of specific growth factor ligands to their cognate receptors activates a pathway involving Ras, and leads to activation of MAPK and PI3K [12,13]. Because of the key role of Ras, this signaling molecule has been a prime target for drug development [14,15]. Major efforts have been directed toward development of farnesyltransferase inhibitors (FTIs). Since Ras must be farnesylated to be anchored in the plasma membrane, the FTIs prevent Ras from localizing in the plasma membrane and result in accelerated degradation in the cytoplasm. While the FTIs exert anti-tumor effects, a body of recent work suggests that mechanisms other than Ras depletion explain the efficacy of these agents [16]. Current speculation is that the FTIs may block Rho B as their primary mechanism of action [16].

Another anti-Ras strategy is to block the binding of Ras to its membrane acceptor sites [17–27]. Ras must be farnesylated and bound to GTP as a pre-requisite for forming a high-affinity complex with membrane acceptor proteins and for its activity in activating the MAPK pathway. Kloog *et al.* have developed a compound capable of dissociating GTP-Ras from its membrane binding sites [17–27]. This agent, called farnesylthiosalicylic acid (FTS), binds specifically to galectin 1 and displaces GTP-Ras from it. As a consequence, GTP-Ras loses its anchor to galectin 1 in the plasma membrane and rapidly

*Current address: School of Animal Studies, University of Queensland, Queensland, Australia

traverses the raft-like structures as well as the non-caveolar regions of the plasma membrane. Lacking an anchor in the membrane, Ras re-enters the cytoplasm where it is degraded and inactivated over a period of several hours. Through this mechanism, FTS interrupts the ability of Ras to signal in the plasma membrane.

While activating mutations of Ras are uncommon in human breast cancer, the MAPK pathway plays a major role in mediating the proliferative effects of estradiol [28]. In addition, the MAPK and PI3K pathways are frequently upregulated in response to estrogen deprivation therapy, and may play a role in the development of hormonal resistance [29–32]. Accordingly, several clinical trials are examining the effect of drugs designed to abrogate Ras effects to prolong the beneficial actions of tamoxifen and the aromatase inhibitors [13].

Several investigators have demonstrated that FTS blocks the activation of MAPK and causes inhibition of the growth of tumors containing activating mutations of Ras (i.e. pancreatic cancer and malignant melanomas) [17–27]. However, no previous studies have examined the effect of FTS on breast cancer because Ras is only infrequently mutated in this neoplasm. However, we reasoned that the frequent upregulation of the MAPK pathway through Ras, which occurs in response to estrogen deprivation therapy, might uncover a role for FTS in this cancer.

The present study examined the effects of FTS on growth of estrogen-dependent breast cancer cells *in vitro* and in xenografts *in vivo*. Since this agent may need to be complexed to solubilizing agents to be orally absorbed, we have examined both free FTS and FTS complexed with cyclodextrin (CD) to make it more water soluble. Herein, we report that the farnesyl analog, FTS, blocks the growth of breast cancer cells in tissue culture and *in vivo*. As an additional, but unexpected, therapeutic advantage, FTS also stimulates cell death. Based upon these observations, we believe that this agent provides a promising drug for further study in women with hormone-dependent breast cancer, either concomitantly with estrogen deprivation therapy or following this strategy.

Materials and methods

FTS and CD were donated by Dr C. Wayne Bardin (Concordia Pharmaceuticals, Bridgewater, New Jersey). The FTS–CD complex was prepared according to instructions from Thyreos. The 'pure' anti-estrogen, ICI 182,780, was donated by Astra-Zeneca (Cheshire, UK). Cell Death Detection ELISA and Cell Proliferation ELISA kits were from Roche (Indianapolis, Indiana), Neutral Red was from Aldrich, improved modified Eagles medium (IMEM) was from Biosource (Camarillo,

California, USA), and FBS was from Gibco (Grand Island, New York, USA). Dextran T70 was from Pharmacia (Piscataway, New Jersey) and Charcoal (NoritA) from Sigma (St Louis, Missouri, USA).

Cell culture

All culture methods have been previously described in detail [29–32]. Briefly, we utilized estrogen receptor-positive MCF-7 breast cancer cells which are called 'wild-type'. When deprived of estradiol over several months, MCF-7 cells develop hypersensitivity to the proliferative and pro-apoptotic effects of estradiol, and display upregulated MAPK activity [29–32]. These cells, described previously in several publications, are termed LTED as an acronym for long-term estradiol deprived [29–32].

Cell number was assayed by the Neutral Red method [33]. We have correlated this method with direct cell counts using a Coulter counter and shown excellent agreement (unpublished data). For all experiments, cells were initially seeded, and allowed to attach and grow for 5 days before estradiol or vehicle was added. Specifically, wild-type MCF-7 cells were initially seeded into 96-well plates in IMEM containing 5% FCS. After a sufficient period of time for the cells to become attached (i.e. 24 h), the medium was changed to that containing 5% charcoal stripped serum (dextran-coated charcoal) in IMEM. The cells were accommodated to this medium for a 5-day period. At this point, the estradiol stimulation component of the studies was initiated. Estradiol and FTS–CD or its vehicle were added in fresh IMEM/5% DCC with a media change after 3 days. Cell number was measured 2 days later (i.e. after 5 days total in estradiol-containing media).

The protocol for LTED cells differed slightly. LTED cells were seeded into 96-well plates and allowed to attach. Twenty-four hours later, the media were changed to IMEM with glutamine added and the cells allowed to grow for 7 days without addition of estradiol. At this time point, the estradiol stimulation protocol was initiated by adding estradiol and FTS–CD or its vehicle at the doses indicated in the figures. In addition, we routinely added 10^{-9} mol/l ICI 182,780 in fresh IMEM to counteract residual estradiol leached from the plastic in the culture dishes. As with the wild-type MCF-7 cells, the media were changed and cells counted 2 days later (i.e. after 5 days total in estradiol-containing media).

It should be noted that our unpublished data indicated that pre-treatment of culture dishes with ethanol removes traces of estrogenic substances which can stimulate the hypersensitive LTED cells. As a practical method to circumvent this problem, small amounts of the pure anti-estrogen ICI 182,780 are added at the time of estradiol. This method has been in use in our laboratory for several years [29–32].

To assay the effects of FTS–CD on cell number, proliferation and apoptosis, cells were seeded into 96-well plates and the next day drug was added in fresh medium. Three days later, fresh drug in medium was added, and cell number, apoptosis and proliferation were measured 2 days later.

Xenografts of long-term estradiol-deprived cells involved castration of nude mice, insertion of silastic implants to clamp plasma estradiol levels at 5 pg/ml and implantation of cells at two sites on the animal. This technique has been described in detail previously [34]. FTS–CD, CD or PBS were administrated daily by i.p. injection for 8 weeks.

Results

Wild-type MCF-7 cells responded to estradiol with maximal stimulation appearing to plateau at a dose of approximately 10^{-11} mol/l which increased cell number nearly 3-fold (Fig. 1a). Increasing concentrations of FTS suppressed estradiol-dependent growth in a dose-responsive fashion with initial inhibition at 50 µmol/l and maximal effects at 75 µmol/l FTS. As a reflection of hypersensitivity to estradiol (as previously extensively described [29–32]), the LTED cells (Fig. 1b) responded maximally to 2 log lower concentrations of estradiol than did wild-type cells (i.e. 10^{-13} versus 10^{-11} mol/l, respectively). In contrast to its effects in wild-type cells, FTS suppressed LTED growth completely at a dose of 25 µmol/l with continued suppression at 50 µmol/l.

Unexpectedly, we observed lesser inhibition of growth at 75 µmol/l FTS in LTED cells.

FTS is a relatively hydrophobic lipid analog and may not be absorbed by patients in an oral formulation. We reasoned that CDs which have been used previously to solubilize hydrophobic drugs might be a practical means to develop a practical formulation of FTS for ultimate use in humans [35]. Accordingly, FTS was complexed with CD and compared to free FTS and to the DMSO vehicle (for free FTS), and to buffer vehicle (for CD-FTS) in our in-vitro system. FTS and FTS–CD exhibited almost identical MCF-7 cell growth inhibition profiles (Fig. 2a). CD alone or buffer vehicle had no significant effect on cell growth of MCF-7 and LTED cells (Fig. 2b and c) under conditions where FTS–CD significantly reduced cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells [30], the degree of inhibition was approximately the same between the two cell lines (Fig. 2b and c). We have also observed similar results in tamoxifen-resistant breast cancer cell lines (data not shown).

Ras-mediated growth factor pathways are required to maintain cellular proliferation and are important regulators of the apoptotic response [36,37]. Reduction of cell number can reflect either an inhibition of proliferation, an enhancement of apoptosis or a combination of these two effects. Accordingly, we systematically examined the effects of FTS specifically on apoptosis and then on

Fig. 1
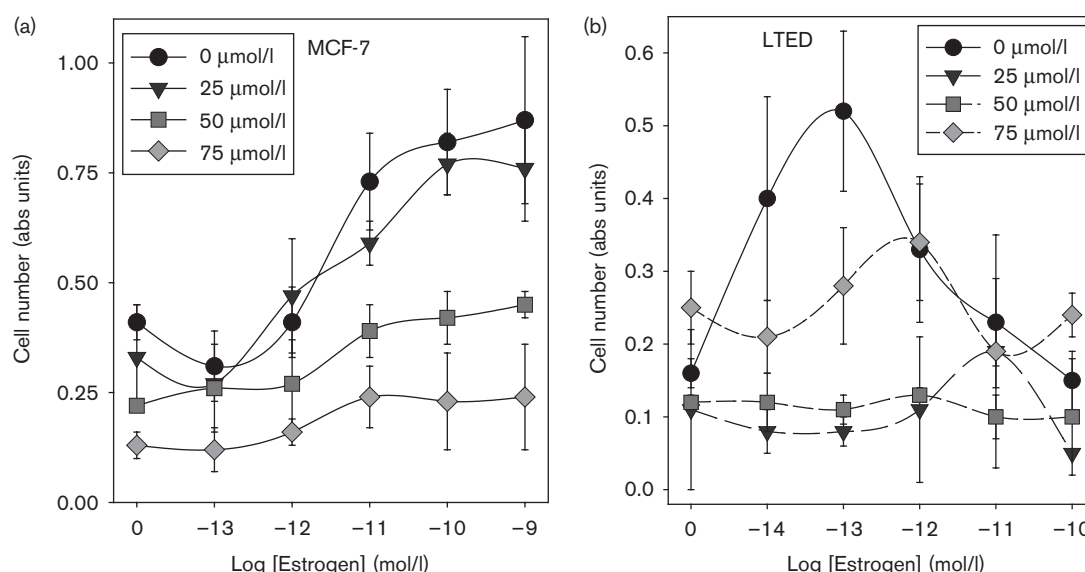
FTS suppresses estradiol-dependent breast cancer cell growth. MCF-7 (a) and LTED (b) cells were grown under conditions where they demonstrate an estradiol response as described in Materials and methods. Increasing concentrations of FTS suppressed this estradiol dependence. Representative of two experiments, means and SDs of four samples are shown. The term (abs units) refers to the absorption units of optical density of the Neutral Red dye used to assess cell number.

Fig. 2
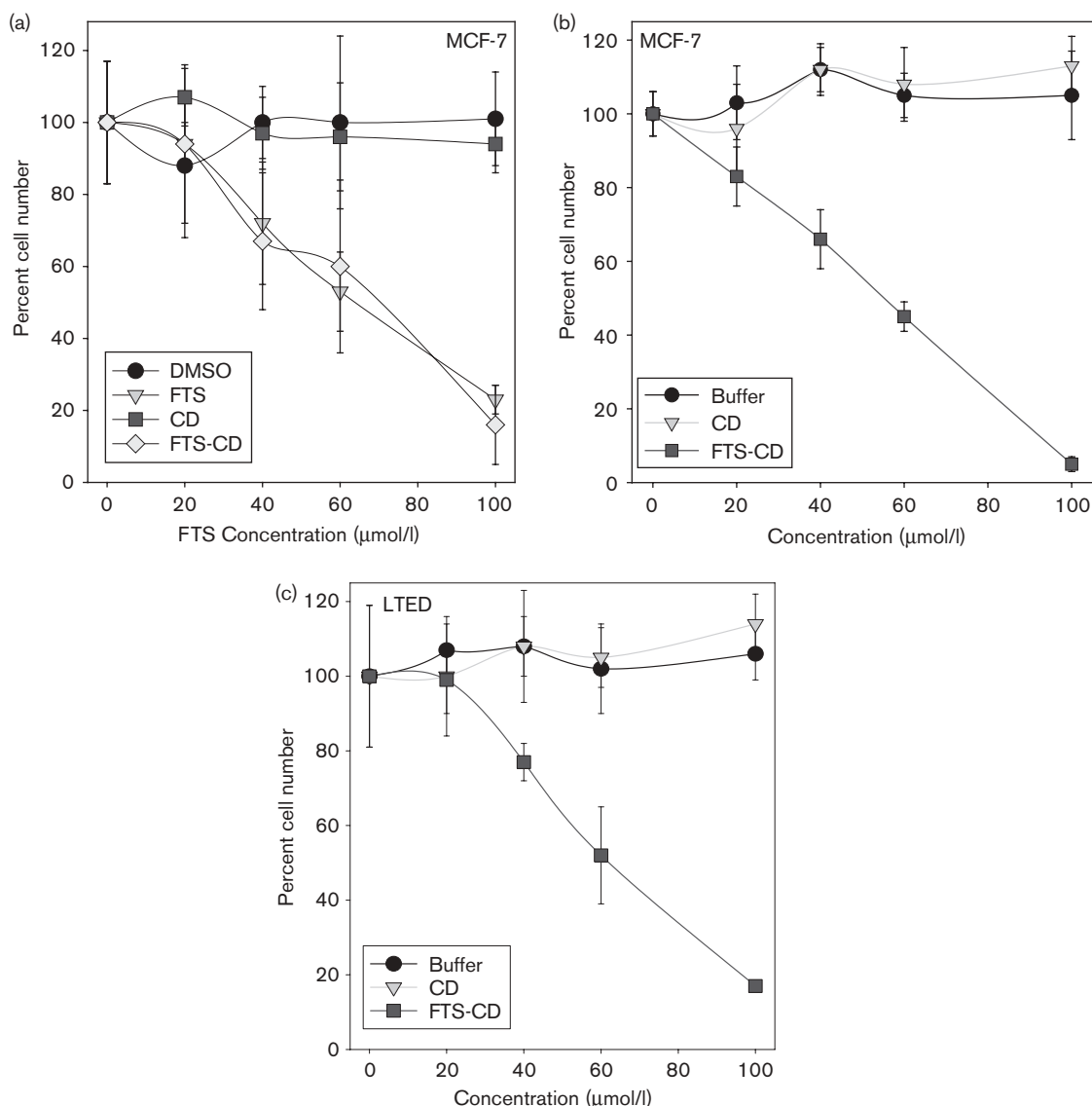
FTS and FTS–CD have similar growth inhibition profiles. (a) MCF-7 cells were grown in the presence of FTS dissolved in DMSO, the equivalent volume of DMSO, FTS–CD dissolved in PBS or the equivalent amount of CD in PBS. Cells were treated for 5 days as described in Materials and methods. Representative of two experiments, means and SDs of four samples are shown. (b and c) FTS–CD inhibits growth of both MCF-7 (b) and LTED (c) cells. Cells were assayed according to the techniques described in Materials and methods. Responses to either PBS, CD in PBS or FTS–CD in PBS added. Representative of six experiments, means and SDs of four samples are shown.

BrdU incorporation as a marker for proliferation. FTS enhanced apoptosis in wild-type and LTED cells starting at a dose of 60 μmol/l and maximally stimulating at a dose of 100 μmol/l FTS–CD to levels 3000- to 4000-fold higher (Fig. 3a and b). Proliferation was also reduced to very low amounts in both cell lines by FTS–CD, but not by CD or buffer alone (Fig. 4a and b). The disruption of both proliferation and apoptosis indicates a global disruption of signaling pathways in breast cancer cells.

In the xenograft model, we compared the administration of vehicle alone, CD alone and CD-complexed with FTS.

CD-FTS caused a statistically significant reduction of tumor weight when measured at the end of the 2-month experiment (Fig. 5).

Discussion
A variety of recent studies have examined the effects of estrogen deprivation therapy on upregulation of growth factor pathways in breast cancer cells [29–32,38–40]. Our group demonstrated a marked upregulation of activated MAPK and of the downstream PI3K effectors, AKT, p70S6 kinase and 4E-BP-1 in MCF-7 cells deprived of estradiol long term [29–32,41,42]. Dowsett *et al.*

Fig. 3
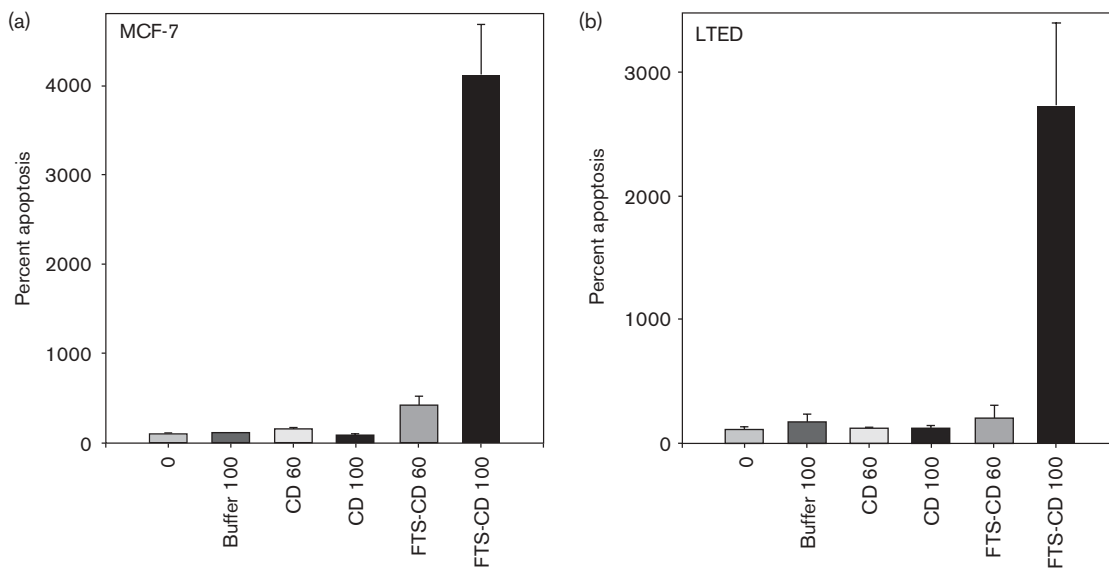
FTS−CD increases apoptosis. MCF-7 (a) and LTED (b) cells were incubated with buffer (equivalent to 100 μmol/l FTS−CD), CD (equivalent to 60 or 100 μmol/l FTS−CD) or 60 or 100 μmol/l FTS−CD for 5 days as described in Materials and methods. Apoptosis was measured by DNA nick site quantitative ELISA. Representative of two experiments, means and SDs of two samples are shown.
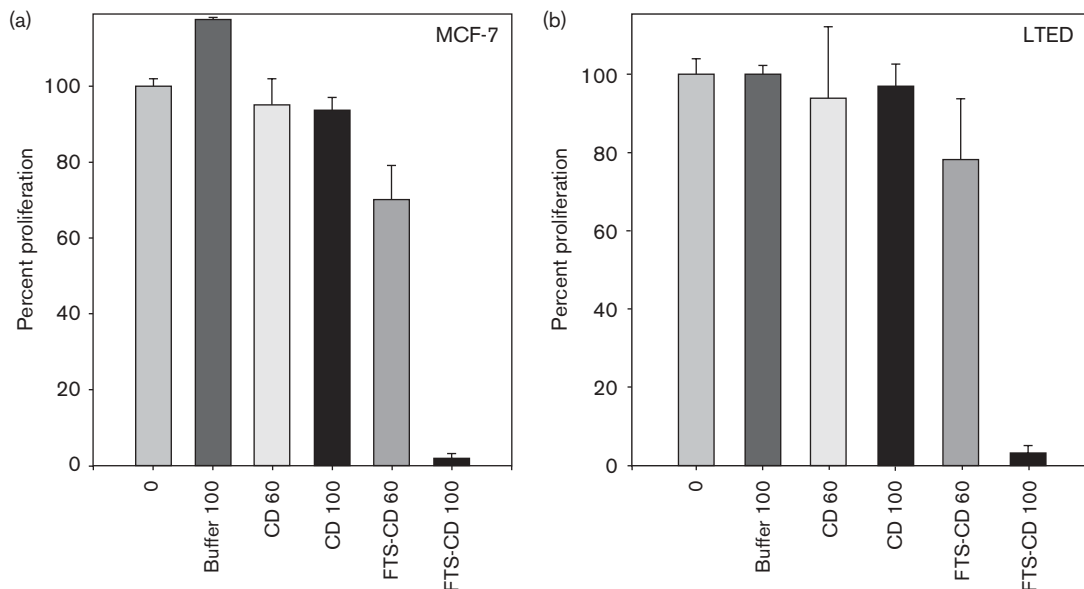
Fig. 4

FTS−CD reduces proliferation. MCF-7 (a) and LTED (b) cells were incubated with buffer, CD or FTS−CD for 5 days as described in Fig. 3. Proliferation was measured by BrdU incorporation. Representative of two experiments, means and SDs of two samples are shown.

confirmed these findings regarding MAPK and also demonstrated an increase in HER-*2* activation [40]. Other investigators also found a marked up-regulation of the MAPK pathway in cells treated with tamoxifen long term [9]. These investigators also demonstrated that blockade of MAPK and of epidermal growth factor receptor pathways in cells subjected to these two forms of estrogen deprivation therapy caused a reduction in cell growth [9]. Based upon these findings, we considered that FTS might provide an effective means of blocking

Fig. 5
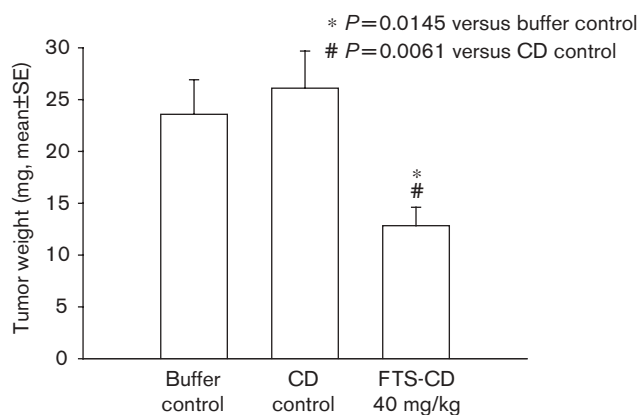
In-vivo effects of FTS–CD on cell growth. LTED cells were implanted into castrated nude mice to form xenografts. Silastic implants delivering estradiol at amounts sufficient to provide plasma levels of estradiol of 5 pg/ml were implanted. One group ($n=9$) received buffer alone (PBS), the second ($n=9$) CD alone and the third ($n=7$) FTS–CD complex (40 mg/kg). Student's t-test was used to compare the effects of FTS–CD to buffer or CD alone.

Ras-mediated growth factor signaling in breast cancer cells. Our results show significant effects of FTS to inhibit cell proliferation and to enhance apoptotic cell death. Based upon these findings, FTS appears to be a candidate drug for testing *in vivo* in xenograft breast cancer models and then in patients.

Our data demonstrated the FTS is active both in cells that have been subjected to estrogen deprivation therapy and in wild-type cells. These results are best explained by the fact that estradiol stimulates proliferation in breast cancer cells through the stimulation of growth factor pathways or through permissive effects that require the growth factor pathways to be active. It would appear then that FTS blocks estradiol-stimulated growth by interrupting these pathways. Although not presented in this paper, our data have shown that FTS blocks MAPK activation in both wild-type and in LTED cells, and the growth of long-term tamoxifen exposed cells. We have also shown that FTS partially blocks AKT activation and more effectively inhibits the activation of p70S6 kinase and 4E-BP-1 [42,43].

Agents such as testosterone are not well absorbed orally and can be rendered orally effective by complexing to CD. Other drugs have also been complexed to CD to enhance solubility and absorption [44,45]. Anticipating the future use of FTS in patients, we examined whether complexing with CD would alter the in-vitro efficacy of FTS. We demonstrated that the FTS–CD complex clearly prevents growth of cellular models of estradiol-dependent (MCF-7) and estradiol-hypersensitive (LTED) breast cancers at the same doses as with free FTS.

FTS–CD also induces apoptosis up to several thousand-fold and reduces proliferation to very low levels.

The observation that FTS–CD suppresses estradiol-dependent growth, but does not shift the dose–response curve to the right as evidence of reduced sensitivity to estradiol, may be significant (Fig. 1a and b). Our prior data indicated that MAPK upregulation in long-term deprived MCF-7 cells causes increased sensitivity to estradiol [29–32]. We have previously demonstrated that inhibition of MAPK with the MEK inhibitor, PD 98059, reverted LTED cells back to the level of estradiol sensitivity observed in wild-type cells [41]. This observation suggests that FTS may exert effects in addition to those mediated by MAPK. This possibility is also supported by the marked suppression of breast cancer cell growth observed. We now have obtained data indicating that FTS is a direct inhibitor of mammalian target of rapamycin (mTOR) [42,43]. In several model systems, mTOR functions as a mediator of cell proliferation. It is possible, therefore, that a major action of FTS is to block mTOR. If correct, FTS would exert effects both on the MAPK and the mTOR pathways. Our recent studies demonstrated upregulation of mTOR in LTED cells [29]. Accordingly, FTS may serve as a unique agent to block more than one signaling pathway involved in breast cancer growth. An additional advantage of FTS is its ability to stimulate apoptosis. Although its mechanism for enhancing apoptosis is not known, recent data suggest that blockade of mTOR can stimulate apoptosis through activation of apoptosis stimulating kinase (ASK)-1 [46,47].

Both FTS and the FTIs were designed to block Ras activity and to inhibit Ras-induced growth in cancer cells. At first consideration, one might consider FTS and the FTIs to be agents in the same class, and to potentially exert similar actions. However, the FTIs are now considered to act through mechanisms other than Ras [16]. FTS exerts unexpected effects to markedly enhance apoptosis however, in addition, recent data demonstrate that FTS also blocks mTOR [42,43]. Taken together, these data strongly support the concept that FTS and the FTIs are not in the same class, and probably will work very differently when administered to patients.

The dose of FTS used in-vitro approaches concentrations that can exert detergent effects. This observation would raise the possibility of non-specific toxic effects of FTS. To address this issue, we examined the effects of a geranyl analog of FTS [*S*-geranylthiosalicylic acid (GTS)] with similar detergent properties to FTS [43]. GTS did not inhibit mTOR, whereas FTS did [43]. This provides evidence of the lack of non-specific detergent effects of FTS. Finally, a wide range of doses of FTS have been used in-vivo in models of pancreatic cancer and malignant melanoma [19–21]. Even a dose of 100 mg/kg causes no

weight loss and no evidence of toxicity in nude mice bearing these tumors. Full toxicology studies are now ongoing in two animal species in preparation for application for an Investigational New Drug Application to perform phase I studies in patients.

In summary, we have shown that FTS and its complexed form, FTS–CD, exert strong anti-proliferative effects on both wild-type and LTED breast cancer cells. An additional action of FTS is to markedly enhance apoptosis. This agent appears to be effective both in breast cancer cells subjected to estrogen deprivation therapy and in wild-type cells. On this basis, FTS might be active as initial treatment of hormone-dependent breast cancer by blocking proliferation and increasing the rate of apoptosis. However, we postulate that FTS may exhibit enhanced activity in patients previously treated with aromatase inhibitors which could result in upregulation of the MAPK pathways. Further in-vivo studies are now required before consideration of use of FTS in phase I studies in women with breast cancer.

References

1 Santen RJ, Manni A, Harvey H, Redmond C. Endocrine treatment of breast cancer in women. *Endocr Rev* 1990; **11**:221–265.
2 Early Breast Cancer Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomized trials. *Lancet* 1998; **351**:1451–1467.
3 Santen RJ. Long term tamoxifen therapy: can an antagonist become an agonist? *J Clin Endocrinol Metab* 1997; **81**:2027–2029.
4 Horwitz KB. When tamoxifen turns bad. *Endocrinology* 1995; **136**:821–823.
5 Ali A, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer* 2002; **2**:101–112.
6 Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res Treat* 1997; **44**:22–38.
7 Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 2003; **9 (1 pt 2)**:447S–4454S.
8 King RJ, Darbre PD. Interaction of growth factors during progression towards steroid independence in T-47-D human breast cancer cells. *J Cell Biochem* 1990; **43**:199–211.
9 Nicholson RI, Hutcheson IR, Knowlden JM, Jones HE, Harper ME, Jordan N, et al. Non-endocrine pathways and endocrine resistance: observations with anti-oestrogens and signal transduction inhibitors in combination. *Clin Cancer Res* 2004; **10**:346s–354s.
10 Nicholson RI, Hutcheson IR, Harper ME, Knowlden JM, Barrow D, McClelland RA. et al. Modulation of epidermal growth factor receptor in endocrine-resistant, oestrogen receptor-positive breast cancer. *Endocr Relat Cancer* 2001; **8**:175–182.
11 Knowlden JM, Hutcheson IR, Jones HE, Madde T, Gee JM, Harper ME, et al. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* 2003; **144**:1032–1044.
12 Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SH, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clin Cancer Res* 2004; **10**:t6S.
13 Johnston SR, Head J, Pancholi S, Detre S, Martin LA, Smith IE, et al. Integration of signal transduction inhibitors with endocrine therapy: an approach to overcoming hormone resistance in breast cancer. *Clin Cancer Res* 2003; **9**:t32S.
14 Joneson T, Bar-Sagi D. Ras effectors and their role in mitogenesis and oncogenesis. *J Mol Med* 1997; **75**:587–593.
15 Dinsmore CJ, Bell IM. Inhibitors of farnesyltransferase and geranylgeranyltransferase-I for antitumor therapy: substrate based design, conformational constraint and biological activity. *Curr Top Med Chem* 2003; **3**:1075–1093.
16 Prendergast GC. Farnesyltransferase inhibitors: antineoplastic mechanism and clinical prospects. *Curr Opin Cell Biol* 2000; **12**:166–173.
17 Elad-Sfadia G, Haklai R, Ballan E, Gabius HJ, Kloog Y. Galectin-1 augments Ras activation and diverts Ras signals to Raf-1 at the expense of phosphoinositide 3-kinase. *J Biol Chem* 2002; **277**:37169–37175.
18 Marom M, Haklai R, Ben-Baruch G, Marciano D, Egozi Y, Kloog Y. Selective inhibition of Ras-dependent cell growth by farnesylthiosalicylic acid. *J Biol Chem* 1995; **270**:22268–22270.
19 Egozi Y, Weisz B, Gana-Weisz M, Ben-Baruch G, Kloog Y. Growth inhibition of ras-dependent tumors in nude mice by a potent ras-dislodging antagonist. *Int J Cancer* 1999; **80**:911–918.
20 Weisz B, Giehl K, Gana-Weisz M, Egoz Y, Ben Baruch, Marciano G, et al. A new functional Ras antagonist inhibits human pancreatic tumor growth in nude mice. *Oncogene* 1999; **18**:2579–2588.
21 Jansen B, Schlagbauer-Wadl H, Kahr H, Heere-Ress E, Mayer BX, et al. Novel Ras antagonist blocks human melanoma growth. *Proc Natl Acad Sci USA* 1999; **96**:14019–14024.
22 Halaschek-Wiener, Wacheck JV, Schlagbauer-Wadl H, Wolff K, Kloog Y, Jansen B. A novel Ras antagonist regulates both oncogenic Ras and the tumor suppressor p53 in colon cancer cells. *Mol Med* 2000; **6**:693–704.
23 Gana-Weisz M, Haklai R, Marciano D, Egozi Y, Ben Baruch G, Kloog Y. The Ras antagonist *S*-farnesylthiosalicylic acid induces inhibition of MAPK activation. *Biochem Biophys Res Commun* 1997; **239**:900–904.
24 Gana-Weisz M, Halaschek-Wiener J, Jansen B, Elad G, Haklai R, Kloog Y. The Ras inhibitor *S-trans,trans*-farnesylthiosalicylic acid chemosensitizes human tumor cells without causing resistance. *Clin Cancer Res* 2002; **8**:555–565.
25 Rotblat B, Niv H, Andre S, Kaltner H, Gabius HJ, Kloog Y. Galectin-1(L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectively inhibits Ras-GTP. *Cancer Res* 2004; **64**:3112–3118.
26 Aharonson Z, Gana-Weisz M, Varsano T, Haklai R, Marciano D, Kloog Y. Stringent structural requirements for anti-Ras activity of *S*-prenyl analogues. *Biochim Biophys Acta* 1998; **1406**:40–50.
27 Elad G, Paz A, Haklai R, Marciano D, Cox A, Kloog Y. Targeting of K-Ras 4B by *S-trans,trans*-farnesyl thiosalicylic acid. *Biochim Biophys Acta* 1999; **1452**:228–242.
28 Pearson G, Robinson F, Gibson TB, Xu BE, Karandikar M, Berman K, et al. Mitogen activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; **22**:153–183.
29 Yue W, Wang JP, Conaway M, Li Y, Santen RJ. Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. *J Steroid Biochem Mol Biol* 2003; **86**:265–274.
30 Jeng MH, Yue W, Eischeid A, Wang JP, Santen RJ. Role of MAPK in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. *Breast Cancer Res Treat* 2000; **62**:167–175.
31 Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, et al. The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol* 2002; **80**:239–256.
32 Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human cancer cells. *J Clin Endocrinol Metab* 1995; **80**:2918–2925.
33 Fiennes AG, Walton J, Winterbourne D, McGlashan D, Hermon-Taylor J. Quantitative correlation of neutral red dye uptake with cell number in human cancer cell cultures. *Cell Biol Int Rep* 1987; **11**:373–378.
34 Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Santen RJ. Estradiol hypersensitivity and MAPK expression in long term deprived human breast cancer cells *in vivo*. *Endocrinology* 2000; **141**:396–405.
35 Pitha J, Gerloczy A, Olivi A. Parenteral hydroperoxypropyl cyclodextrins: intravenous and intracerebral administration of lipophiles. *J Pharm Sci* 1994; **83**:833–837.
36 Sebolt-Leopold JS. MEK inhibitors: a therapeutic approach to targeting the Ras-MAPK pathway in tumors. *Curr Pharm Des* 2004; **10**:1907–1914.
37 Cox AD, Der CJ. The dark side of Ras: regulation of apoptosis. *Oncogene* 2003; **22**:8999–9006.
38 Daly RJ, Darbre PD. Cellular and molecular events in loss of estrogen sensitivity in ZR-75-1 and T-47-D human breast cancer cells. *Cancer Res* 1990; **50**:5868–5875.
39 Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* 1993; **53**:283–290.
40 Martin LA, Farmer I, Johnston SR, Ali S, Marshall C, Dowsett M. Enhanced estrogen receptor (ER) alpha, ERBB2, and MAPK signal transduction

pathways operate during the adaptation of MCF-7 cells to long term estrogen deprivation. *J Biol Chem* 2003; **278**:30458–30468.

41 Yue W, Wang JP, Conaway M, Masamura S, Li Y, Santen RJ. Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. *Endocrinology* 2002; **143**:3221–3229.

42 Yue W, Wang JP, Li Y, Fan P, Santen RJ. Farnesylthiosalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. *Int J Cancer* 2005; **117**:746–754.

43 McMahon LP, Yue W, Santen RJ, Lawrence Jr. JC. Farnesylthiosalicylic acid inhibits mTOR activity both in cells and *in vitro* by promoting dissociation of the mTOR–raptor complex. *Mol Endocrinol* 2005; **19**:175–183.

44 Pitha J, Irie T, Sklar PB, Nye JS. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. *Life Sci* 1988; **43**:493–502.

45 Stella VJ, Rajewsk RA. Cyclodextrins: their future in drug formulation and delivery. *Pharm Res* 1997; **14**:556–557.

46 Huang S, Shu L, Dilling MB, Easton J, Harwood FC, Ichijo H, *et al.* Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21^{Cip1}. *Mol Cell* 2003; **11**:1491–1501.

47 Matsuura H, Nishitoh H, Takeda K, Matsuzawa A, Amagasa T, Ito M, *et al.* Phosphorylation-dependent scaffolding role of JSAP1/JIP3 in the ASK1–JNK signaling pathway. A new mode of regulation of the MAPK cascade. *J Biol Chem* 2002; **77**:40703–40709.